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Are long corolla tubes in *Pedicularis* driven by pollinator selection?

Summary The evolution of long corolla tubes has been hypothesized to be driven by long-tongued pollinators. Corolla tubes in *Pedicularis* species can be longer than 10 cm which may function as flower stalks to increase visual attractiveness to pollinators because these species provide no nectar and are pollinated by bumblebees. The corolla tube length was manipulated (shorter or longer) in two *Pedicularis* species in field to examine whether longer tubes are more attractive to pollinators and produce more seeds than short tubes. Our results did not support the pollinator attraction hypothesis, leaving the evolution of long tubes in *Pedicularis* remains mysterious.

The hypothesis that the evolution of long corolla tubes is driven by the mechanical fit with long-tongued pollinators has been proposed since Darwin (Pauw et al. 2009; Muchhala and Thomson 2009; Newman et al. 2015). Plants with longer corolla tubes are more likely to fit with long-tongued pollinators and consequently achieve higher reproduction than plants with shorter corolla tubes, and such directional selection may favor long corolla tubes in a population. Experimental reduction of the corolla tube length reduced reproductive success both in *Platanthera* (Nilsson 1988) and in *Disa* (Johnson and Steiner 1997), supporting Darwin's model of pollinator-mediated selection. While these studies demonstrate modification of floral shape by pollinator-mediated selection, it remains unclear how flowers diverge in related plants pollinated by similar types of animals (Wilson and Thomson 1996).

The center of *Pedicularis* (Orobanchaceae, formerly Scrophulariaceae) diversity is in the Himalayan area where more than 300 species exhibit great interspecific variation in corolla morphology, with the straight corolla tube ranging in length from less than 1 cm to over 10 cm (Yu et al. 2015). Previous studies indicate that both long- and short-tubed *Pedicularis* species are pollinated by bumblebees (Macior and Sood 1991; Huang and Shi 2013; Liu et al. 2016), but the proposed long-tongued pollinators (Lepidoptera) have not been observed perhaps because long-tubed *Pedicularis* species do not provide nectar reward (Liu et al. 2015). Thus, long-tubed *Pedicularis* species that occur only in the Himalayan area provide an exceptional plant group in which to examine how flowers diverge without a shift of major pollinators.

To explain long corolla tubes in *Pedicularis* species, Lazarus Walter Macior, an expert in the pollination biology of this genus, proposed that long corolla tubes may function as flower stalks to increase visual attractiveness to pollinators by raising flowers a greater distance above the leaves (Macior and Sood 1991), but this pollinator attraction hypothesis has not been empirically examined. To test Macior's hypothesis, we manipulated the corolla length of two *Pedicularis* species in the field populations at Shangri-La Alpine Botanical Garden, southwest China (Doc S1). Corolla tube length in *P. tricolor* (4–6 cm) was shortened to 2–3 cm. In *P. siphonantha* corolla tube lengths from less than 3 cm to over 7 cm were created by transplanting seedlings into different watering and sunlight conditions. Corolla tube length was manipulated (shorter or longer) to examine whether longer tubes are more attractive to pollinators and produce more seeds than short tubes.

We observed that one bumblebee species, *Bombus friseanus* Skorikov, was the most frequent floral visitor (Figure 1) for the two *Pedicularis* species, with occasional visits by *B. festivus* Smith. In *P. tricolor* we observed bumblebee visits to 12 flowering individuals in three sunny days (August 3, 6 and 12), the total numbers of first arrivals to 72 corolla-tube-shortened flowers and 72 unmanipulated flowers were 89 and 86, suggesting that bumblebee pollinators did not prefer long-tubed over short-tubed flowers. Visits per census to shortened (Mean $\pm$ *SE*, 2.70 ± 0.34) and unmanipulated flowers (2.61 ± 0.37) were not significantly different ($F_{1,\ 64} = 0.033$, $P = 0.857$). Seed set per capsule of shortened (0.45 ± 0.022) and unmanipulated flowers (0.48 ± 0.018) was not significantly different ($F_{1,\ 180} = 1.264$, $P = 0.262$). The mean corolla length of open-pollinated flowers was 52.19 ± 0.62 mm and their corresponding seed set per flower was 0.29 ± 0.02. Among these 100 open-pollinated flowers in *P. tricolor*, there was no significant correlation between corolla tube length and seed set ($P = 0.470$).

In *P. siphonantha* seedlings under watering and shading treatment yielded obviously longer corolla tubes in some flowering individuals (Figure 2). The corolla tube length of the short-tubed group (43.12 ± 1.48 mm, $n = 12$ plants) was significantly less ($F_{1,\ 23} = 57.51$, $P < 0.001$) than that of long-tubed group (59.75 ± 1.60 mm, $n = 13$). We found that longer corolla tubes did not attract more pollinator visits. Neither numbers of bumblebee visits per plant ($R = 0.272$, $P = 0.189$, $n = 25$) nor visitation frequency ($R = 0.276$, $P = 0.181$) was significantly correlated with corolla tube length (Figure 2). The effect of corolla tube length on seed set per flower (0.41 ± 0.06) was not significant ($P = 0.065$), but there was a positive correlation between visitation frequency (1.89 ± 0.29) and seed set ($R = 0.408$, $P = 0.043$). The corolla tube length of open-pollinated flowers (41.01 ± 1.09 mm) was not significantly correlated with seed set per flower (0.35 ± 0.04) ($R = 0.087$, $P = 0.569$, $n = 45$).

Previous studies shortening the corolla tubes showed that plants possessing slightly longer corolla tubes had a reproductive advantage because pollination efficiency was increased when long-tongued pollinators probed nectar at the bottom of the corolla, increasing their contact with anthers and stigmas (Nilsson 1988; Johnson and Steiner 1997). In a hawkmoth-pollinated iris (*Gladiolus longicollis*), Alexandersson and Johnson (2002) showed that inflorescence height and tube length were positively correlated with both fruit and seed set. Plants with shorter tubes were not effectively pollinated because of their mismatch with the tongue lengths of the major pollinator. Floral tube length of a long-tongued fly pollinated iris was positively correlated with pollen receipt, suggesting that an increase of tube length increased its female fitness (Pauw

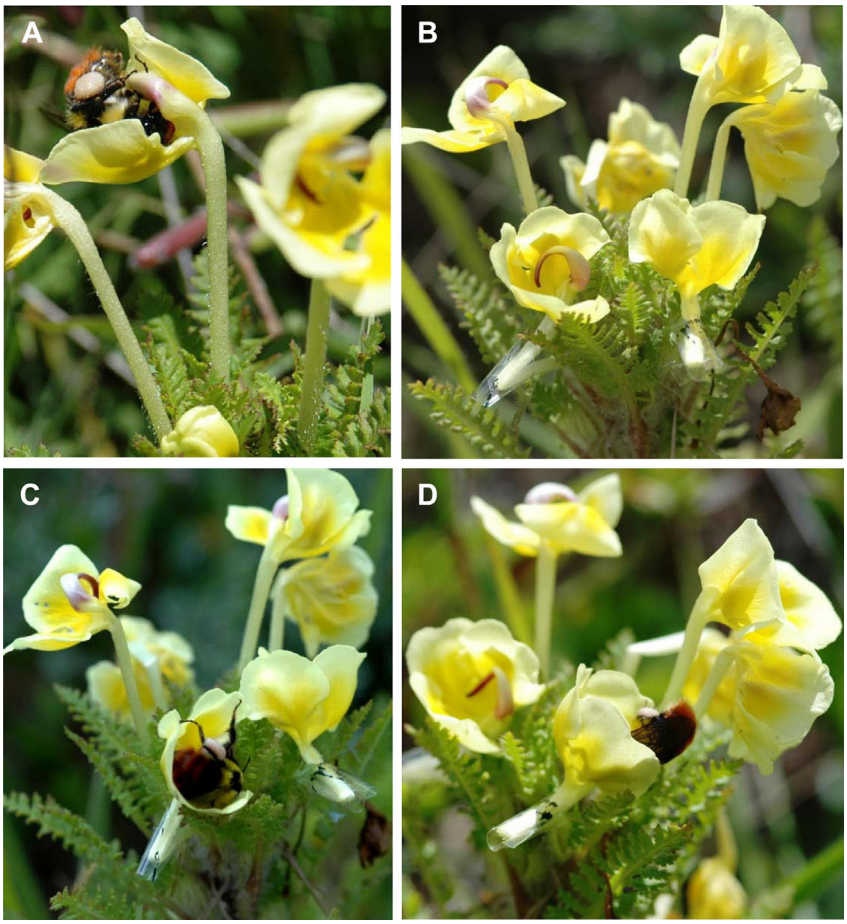

Figure 1. One bumblebee species *Bombus friseanus* visiting corolla-tube-shortened and unmanipulated flowers of *Pedicularis tricolor* in an experimental plot
A bumblebee collecting pollen from an unmanipulated long-corolla-tubed flower **(A)**; two flowers in which the corolla tube has been shortened by bending the tubes and fixing them with clear sticky tape **(B)**; a bumblebee visiting the left hand **(C)** and the right hand **(D)** manipulated flower of the individual in **(B)**.

et al. 2009). Given that no long-tongued pollinators were observed in *Pedicularis*, Macior and Sood (1991) proposed that the long corolla could function to increase visual attractiveness. Our observations on *P. tricolor* with shortened corolla tubes and *P. siphonantha* with longer corolla tubes in experimental plots did not reveal any preference by bumblebees for long corolla tubes over short ones. Correspondingly, in the two species seed set of manipulated flowers did not decrease or increase significantly compared to control flowers. Consistent with this result, variation in corolla tube length of unmanipulated open-pollinated flowers was not related to seed production in either species. Our findings do not support the pollinator attraction hypothesis.

Numerous flowers under natural pollination did not produce any seeds in the two *Pedicularis* species. This is why seed set of open-pollinated flowers was lower than that of manipulated flowers which aborted fruits (no seeds) were excluded, suggesting that both species experienced pollen limited seed set. The pollinator attraction hypothesis predicts that seed set should be higher in longer-corolla-tubed flowers than in shorter ones, given that the former attract more pollinator visits. However, we did not observe a correlation between corolla tube length and seed set per flower in open-pollinated flowers of either species.

This pollinator attraction hypothesis conflicts with some *Pedicularis* species which produce tall stems but also have long corolla tubes; their flowers are conspicuous and not hidden by leaves. The repeated parallel evolution of long-tubed species from ancestral short-tubed species suggests that long corolla tubes associated with loss of nectar are adaptive (Yu et al. 2015). The functional significance of long corolla tube in disparate lineages of *Pedicularis* species is still a mystery. Long corolla tubes and the associated long styles may be more effective in eliminating heterospecific or unfit pollen than shorter corolla tubes and styles, i.e., filtering pollen interference hypothesis, but experimental evidence remains unknown.

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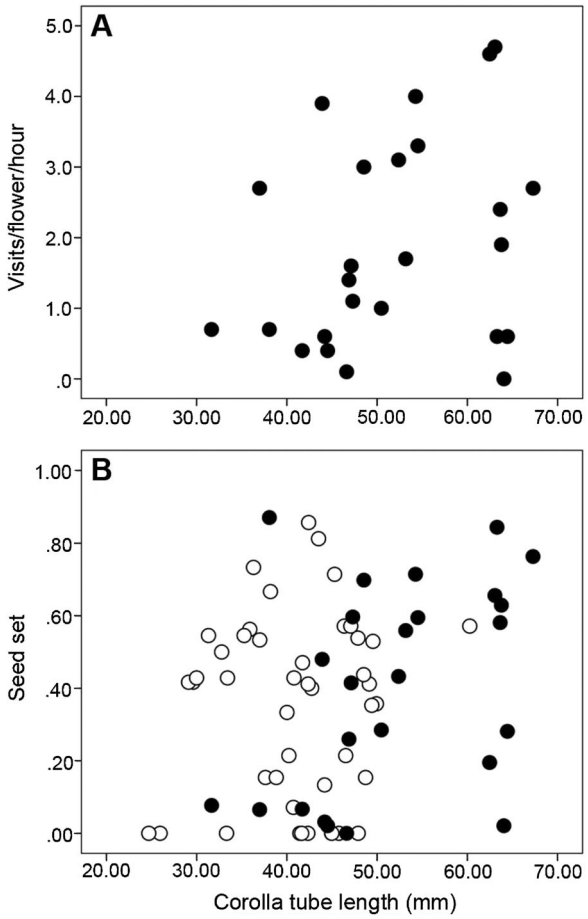

Figure 2. No correlation between corolla tube length and bumblebee visitation frequency (A) and seed set (B) in *Pedicularis siphonantha*

The number of bumblebee visits per flower to 25 plants in the experimental plot was not significantly correlated with corolla tube length ($P = 0.181$). Seed set per flower of the 25 plants in the experimental plot which individuals grew longer corolla tubes under watering and shading (closed circles, $P = 0.065$) and of 45 open-pollinated flowers (open circles, $P = 0.569$) were not significantly correlated with corolla tube length.

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Shuang-Quan Huang[1*], Xiang-Ping Wang[2] and Shi-Guo Sun[1]
[1]School of Life Sciences, Central China Normal University Wuhan 430079, China
[2]College of Life Sciences, Wuhan University Wuhan 430072, China
*Correspondence: hsq@mail.ccnu.edu.cn

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S-Q.H. and X-P.W. performed most of the research and S-Q.H. drafted the manuscript. S-G.S. joined wadding and watering experiments and observed in the field, all authors discussed, designed the experiment, and revised the manuscript

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